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AWARD NUMBER DAMD17-96-1-6249

TITLE: Role of HMG-I (I) in Human Breast Cancer Metastasis

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (24 Jun 97 - 23 Jun 98)

4. TITLE AND SUBTITLE
Role of HMG-I (Y) in Human Breast Cancer Metastatis

5. FUNDING NUMBERS
DAMD17-96-1-6249

6. AUTHOR(S)
Ying Li

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington State University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19981210 096

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

HMG-I(Y) proteins are mammalian architectural transcription factors that regulate transcription of a number of genes. The original proposal is to test a hypothesis that expression of HMG-I(Y) proteins will fundamentally influence the expression of genes, which are involved in metastatic invasion, migration, angiogenesis and colonization. In the second year of study, a great effort has been made to isolate HMG-I(Y) high-expressing breast cancer cell transfectants. These HMG-I(Y) transfectants and HMG-I(Y) monitoring systems are essential tools for further study of metastatic potential and expression of down-stream genes in HMG-I(Y) transfectants. Two new approaches have been developed. (a) HA-tagged HMG-I(Y) gene constructs were generated. Isolation of stable HMG-I(Y)-expressing clones, including dominant negative HMG-I(Y) clones, by using monoclonal antibody against HA-tag is in progress. (b) HA-tagged HMG-I(Y) genes were subcloned into a tetracycline inducible gene expression system. The preliminary results indicated that the exogenous HMG-I(Y) proteins in MCF-7 cells could be increased to 50-fold after tetracycline induction.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, High Mobility Group Protein HMG-I(Y), Transcription Factor, Metastasis	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

High mobility group proteins I and Y [HMG-I(Y)] are mammalian architectural transcription (1). They have been demonstrated to be required for the transcription regulation of a number of genes, including the genes associated with tumorigenesis and metastasis (1-3). Furthermore, the expression of HMG-I(Y) is correlated with tumor progression (4). The funded proposal is to define the role of HMG-I(Y) in tumorigenesis and metastasis. The experimental strategies of the proposed work are: (I) over-expression of HMG-I(Y) in non-metastatic tumor cell lines and over-expression of dominant negative HMG-I(Y) or anti-sense HMG-I(Y) in highly metastatic tumor cell lines; (II) examination of the characteristic changes of these transfectants, including the down-stream genes of HMG-I(Y) that are involved in metastatic invasion, migration, angiogenesis, and colonization.

In the first annual report, the Task1 to 4 of the proposal are completed or initiated. They are: (a) construction and characterization of mammalian expression vectors that contain sense, anti-sense, or dominant negative human HMG-I(Y) genes; (b) expression of HMG-I(Y) proteins in MCF-7 and P-/P+ cell systems; and (c) regulation of metastasis-associated stromelysis (MMP-3) gene by HMG-I(Y); and (d) phosphrylation of HMG-I(Y) proteins by protein kinases C *in vitro* and *in vivo*. These preliminary results support the hypothesis of this proposal and a part of these data has been published on scientific journal (5).

In this second annual report, the studies from expression of antisense HMG-I(Y) in MCF-7 and Hs578T further support the hypothesis. However, assays for metastatic potential and expression of down-stream genes in HMG-I(Y) transfectants have been hampered due to low-level expression of exogenous HMG-I(Y) and lack of means to monitor the expression of exogenous HMG-I(Y) proteins in transfected tumor cells. Two alternative approaches have recently been established to solve these technical difficulties, that is, expression of tagged HMG-I(Y) proteins and a tetracycline inducible expression system. The preliminary results indicated a more than 50-fold increase of exogenous HMG-I protein in MCF-7 transfectants after induction. Isolation of high expressing clones and assays for metastatic potential are now in progress.

RESULTS AND DISCUSSION

(1) Expression of antisense HMG-I(Y) and the effect on cell growth

The mammalian expression vector encoded antisense HMG-I (12, 13) was transfected into the non-metastatic human breast cancer cell line MCF-7 and high-metastatic cell lines Hs578T and HeLa. The pool of these transfectants was selected by G418 for a week. The G418-resistant cells were examined by an *in vitro* growth assay. As shown in **Figure 1**, expression of antisense HMG-I inhibits the growth of MCF-7 and Hs578T cells, but not the growth of HeLa cells. The different inhibition may be

due to the different expression of endogenous HMG-I(Y) because the endogenous HMG-I(Y) proteins in HeLa cells is about 10 fold higher than that in Hs578T cells and about 20-25 fold higher than that in MCF-7 cells **(Figure 2)**. The data suggest that the antisense DNA may be insufficient to suppress the growth of tumors that express high levels of HMG-I(Y) proteins. The results agree with the earlier report (6).

The MCF-7 cells transfected with antisense HMG-I were also examined by soft agar assay. The result demonstrated that expression of antisense HMG-I leads to a significant reduction of colony formation **(Table 1).**

Table 1. Antisense of HMG-I inhibits the growth of MCF-7 cells in soft agar

	No. of cell seeded	Ave. of clones	clone efficiency (%)
MCF-7/vector	1000	25	2.5
MCF-7/anti-I	1000	6	0.6

(2) **Expression of exogenous HMG-I(Y) proteins**

Assays for metastatic potential and expression of down-stream genes in HMG-I(Y) transfectants have been hampered due to low expression of exogenous HMG-I(Y) proteins and lack of means to monitor the expression of exogenous HMG-I(Y) proteins in transfected cells. In the previous report, pools of MCF-7 cells that transfected with cDNA of human HMG-I or Y grow more and larger colonies than those transfected with empty vector in soft agar. However, the HMG-I(Y) proteins (endogenous and exogenous) of HMG-I(Y) transfectants are about 1-1.5 fold higher than those of empty-vector transfectants, suggesting the low expression of exogenous HMG-I(Y) proteins. Isolation of high-expressing clones has been attempted by screening expression of HMG-I(Y) proteins in individual transfected clones. The problem is that the anti-HMG-I(Y) polyclonal antibodies, which have been generated and used for HMG-I(Y) detection in our laboratory, cannot distinguish the transfected exogenous HMG-I(Y) proteins from the endogenous ones. The problem is particularly obvious for exogenous HMG-I(Y) expression in dominant negative transfectants. In addition, the polyclonal antibodies can only be used for Western blot but not for immunoprecipitation. It is difficult to screening clones with Western blot alone.

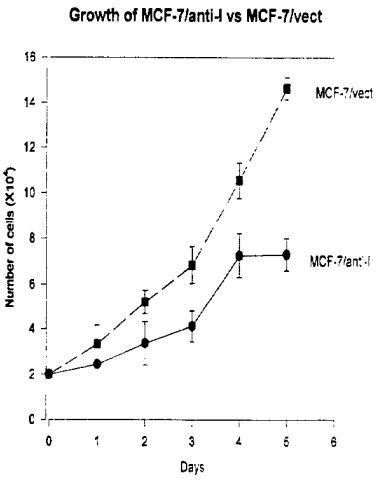
To overcome these problems, expression of tagged HMG-I(Y) proteins and an inducible expression system were established.

First, the constructs that encode a 9-amino acid peptide from hemagglutinin (HA tag) fused to the N-terminal end of HMG-I and HMG-Y were generated. This HA tag was used intensively in the study of BRCA1 and other cell cycle related proteins. It has been reported that these HA tagged proteins have no difference from the untagged proteins in DNA binding and interaction with other nuclear proteins (7,8). Monoclonal

antibody 12CA5 specifically against HA-tag can be used to perform both immunoprecipitation and Western blot. This method makes it possible to screen individual clones for high HMG-I(Y) expression. Furthermore, it provides a way to distinguish exogenous HMG-I(Y) from endogenous HMG-I(Y), especially to distinguish dominant negative HMG-I(Y) from endogenous HMG-I(Y).

Second, a tetracycline inducible gene expression system has been adopted, which offers tightly regulated, high-level gene expression (9). HA-tagged HMG-I and HMG-Y have been subcloned into a tetracycline inducible vector (*tet-off*) and transfected into MCF-7 cells. The expression of HMG-I(Y) in transfectants can be manipulated by culturing them in the presence or absence of tetracycline. As shown in **Figure 3**, the exogenous HMG-I protein in the induced MCF-7 cells was upregulated about 50-fold than that in non-induced MCF-7 cells. The test for metastasis potential between the induced and the non-induced MCF-7 cells is in progress. The inducible expression system also provide an opportunity to study the expression of down-stream genes, which are response to the stimulation.

A



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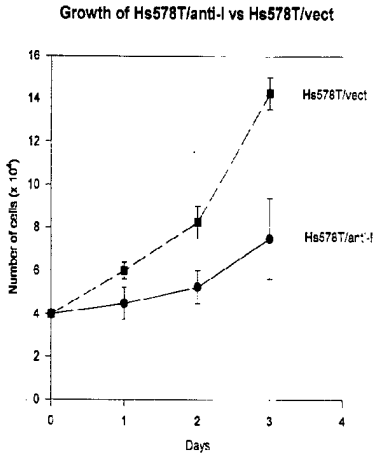

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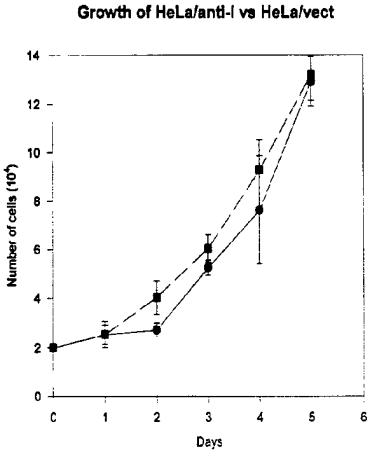

Figure 1: Different growth rates between transfectants of antisense HMG-I and transfectants of empty vector in human breast cancer cell lines MCF-7 and Hs578T. MCF-7 (**A**), Hs578T (**B**) and HeLa (**C**) cells were transfected with antisense HMG-I or empty vector. The pools of transfectants were selected by G418 for a week. The 2-4 x 10^4 cells in 10% FCS MEM medium in the presence of G418 were incubated at 37°C and 5% CO_2 in humidified incubator. Trypan blue-resistant cells were counted and each of the points represents average number of the cells from four cell-culture dishes.

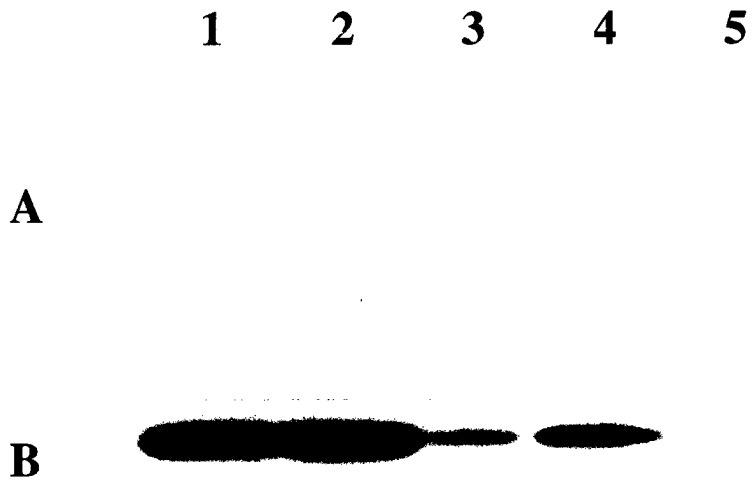

Figure 2: Endogenous HMG-I(Y) proteins in different human cell lines.
A relative amount of total extracted proteins was run on SDS-PAGE and transferred onto cellulose membrane. Histone H1 on the membrane was stained with Coomassie blue (**A**). HMG-I(Y) were detected by Western blot with a polyclonal antibody against human HMG-I(Y) (**B**). In this case, Lane 1 is from highly metastatic HeLa cells; Lane 2 from highly metastatic MCF-7/PKCα cells; Lane 3 from non-metastatic MCF-7; Lane 4 from highly metastatic Hs578T cells; and Lane 5 from normal epithelial Hs578Bst cells.

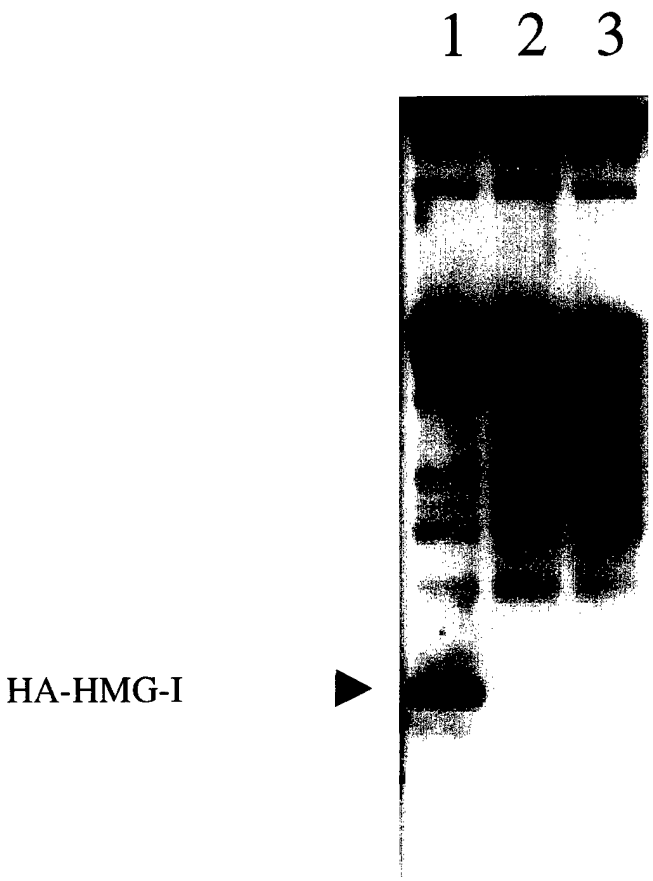

Figure 3: Expression of HA-tagged HMG-I protein in *on* or *off* condition of tetracycline inducible expression system. MCF-7 cells are transfected with HA-tagged HMG-I *tet-off* construct or with mock transfection. The transfectants were cultured in the absence of tetracycline (**Lane 1. *tet-on*, induced**) or in the presence of 2 μg/ml of tetracycline (**Lane 2: *tet-off*, non-induced**) for 48 hours. The mock transfectants were cultured in the absence of tetracycline (**Lane 3: mock**). 5 x 10^6 cells were lysed and immunoprecipitated with antibody 12CA5 specifically against HA tag. Then the HA-tagged HMG-I(Y) proteins were detected by Western blot with antibody MR18 specifically against HMG-I(Y).

CONCLUSIONS

In the second year of study, considerable progress has been made after almost six months of the frustration in isolation of exogenouse HMG-I(Y) high-expressing MCF-7 cells. Two alternative approaches have been developed. First, mammalian expression vectors that contain HA-tagged HMG-I(Y) genes have been constructed. The immunoprecipitation and Western blot by using monoclonal antibody against HA-tag offers an effective method to screen HMG-I(Y)-expressing clones and distinguish exogenous HMG-I(Y) from endogenous ones. Isolation of high-expressing clones from the transfectants encoded HA-tagged HMG-I(Y) genes now is in progress. Second, HA-tagged HMG-I(Y) genes have been subcloned into a tetracycline inducible gene expression system. The preliminary results indicated that the exogenous HMG-I(Y) proteins in MCF-7 cells could be increased to 50-fold after tetracycline induction. With the help of these new techniques, I should be able to monitor metastatic potential and expression of down-stream genes in HMG-I(Y) transfectants and complete the study on role of HMG-I(Y) in human breast cancer metastasis during the last year of the funding period.

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